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Genetic Disruption of T Cell Tolerance Mechanisms

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<b>13. Abstract (Maximum 200 Words)</b> <i>(abstract should contain no proprietary or confidential information)</i> <p>We are developing a mouse model for ovarian cancer that will allow monitoring of the <i>in vivo</i> activities of tumor-specific T cell clones as they encounter ovarian tumors <i>in vivo</i>. We proposed to "tag" the <i>neu</i> oncogene with two defined T cell epitopes so as to confer recognition by available T cell receptor (TCR) transgenic T cells. When expressed in the murine ovarian tumor cell line ID8, epitope-tagged <i>neu</i> (designated <i>neu</i><sup>OT1/OT2</sup>) should induce formation of aggressive ovarian adenocarcinomas that express the epitope tags and hence are recognizable by adoptively transferred TCR transgenic T cells. We successfully made the <i>neu</i><sup>OT1/OT2</sup> expression construct, but found it to be overly immunogenic <i>in vivo</i> such that tumors were spontaneously rejected. Therefore, we derived a third generation ID8 tumor cell line that has a shorter tumor latency and decreased expression of MHC Class I, which should make it less immunogenic. Meanwhile, we have commenced adoptive T cell transfer experiments using a convenient, transplantable lymphoma model, and have discovered signaling differences between T cells that are responding to antigen-positive tumors versus the same antigen delivered with adjuvant. Finally, Cbl-b <sup>-/-</sup> mice have been obtained and are currently being backcrossed onto the B6 background for Aim 3.</p>				
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## **DAMD17-01-1-0733 Annual Progress Report**

**PI:** Brad H. Nelson, Ph.D.

**Title of Project:** Eliciting Autoimmunity to Ovarian Tumors in Mice by Genetic Disruption of T Cell Tolerance Mechanisms

### **Introduction:**

Research in the fields of basic immunology and autoimmunity has identified several distinct mechanisms through which immune tolerance is established and maintained in the normal host, and additional mechanisms will likely be identified in future. We hypothesize that ovarian tumors are recognized in an antigen-specific manner by T cells but induce immunologic tolerance through one or more of these homeostatic mechanisms, which have evolved to protect the host from autoimmune attack. We further hypothesize that tolerance to ovarian tumors can be overcome by disrupting critical components of tolerogenic pathways through genetic manipulation of T cells. To test this hypothesis, we proposed to develop a murine model for ovarian cancer that will allow, for the first time, precise monitoring of the functional responses of naïve, tumor-specific CD4+ and CD8+ T cell clones to ovarian tumors. Multiple properties of tumor-reactive T cells will be assessed *in vivo*, including their localization, activation, anergic status, proliferation and apoptosis. Differential responses and anti-tumor activities of the CD4+ and CD8+ T cell subsets will be investigated. Finally, the model will be used to evaluate the functional responses of tumor-specific CD4+ and CD8+ T cells that are genetically pre-disposed to autoimmune activity. The first tolerogenic pathway tested will be that involving the Cbl-b gene, as T cells lacking Cbl-b have a greatly reduced requirement for CD28 co-stimulation and demonstrate hyperactivity *in vivo* with profound autoimmune sequelae. The specific aims of this proposal are:

- Aim 1. To generate an ovarian tumor cell line that is recognized by antigen-specific CD4+ and CD8+ T cell clones from TCR transgenic mice.
- Aim 2. To define the mechanisms by which ID8 ovarian tumors evade rejection by tumor-specific CD4+ and CD8+ T cells.
- Aim 3. To determine whether tumor-specific CD4+ and CD8+ T cells lacking the Cbl-b gene show enhanced functional responses to ovarian tumors.

### **Body:**

#### **Aim 1: To generate an ovarian tumor cell line that is recognized by antigen-specific CD4+ and CD8+ T cell clones from TCR transgenic mice.**

We proposed to use the ID8 OSE ovarian tumor cell line derived by Dr. Paul Terranova's group, and to stably transfect this cell line with a construct called *neu*<sup>OT1/OT2</sup>. This was accomplished, and high levels of expression of *neu*<sup>OT1/OT2</sup> were achieved. Furthermore, using the proliferative assay described in section E.1.2 of the proposal, we showed that the OT1 and OT2 T cell epitopes were presented to CD8+ and CD4+ T cells, respectively (Figure 1). As expected, expression of MHC Class I was retained, whereas the cells remained negative for MHC Class II, B7-1, B7-2, Fas and FasL (not shown).

As per the proposal, this modified cell line was then injected i.p. into female B6 mice. Unfortunately, the cells no longer induced tumors. Untransfected control ID8 tumor cells, or cells transfected to express untagged *neu*, induced advanced tumors after approximately 120 days. By contrast, ID8 cells expressing *neu*<sup>OT1/OT2</sup> failed to induce tumors even after one year. We concluded that the OT1 and OT2 epitope tags caused the tumor cells to be sufficiently immunogenic that they were spontaneously rejected.

To circumvent this problem, we have generated new ID8 subclones that are more aggressive and less immunogenic such that they give rise to tumors within 30-40 days. This was done by serially transplanting non-transfected ID8 tumor cells into female mice. While first generation tumors take ~120 days to arise, if these tumors are excised, cultured briefly, and injected into new host mice, the second generation tumors take only 50-60 days to arise. If this process is repeated once more, the third generation tumors arise in just 30-40 days.

These third generation tumors are indistinguishable from the original ID8 cells by light microscopy, and grow at approximately the same rate in vitro. However, they show reduced expression of MHC Class I, and may well have other molecular changes that contribute to their more aggressive phenotype. In vivo, they form tumors that resemble those of the original ID8 cell line in that they disseminate throughout the peritoneal cavity, implant on multiple abdominal organs, and induce formation of bloody ascites.

We are currently transfecting several subclones of third generation ID8 tumor cells with the *neu*<sup>OT1/OT2</sup> vector. We will select several stable transfectants that by flow cytometry show high, medium, or low expression of *neu*<sup>OT1/OT2</sup>. These will be injected i.p. into multiple female B6 mice, and we will then measure the latency of tumor formation. The subclone that gives the shortest tumor latency while still retaining expression of *neu*<sup>OT1/OT2</sup> and MHC Class I will be used for all subsequent studies.

**Aim 2: To define the mechanisms by which ID8 ovarian tumors evade rejection by tumor-specific CD4+ and CD8+ T cells.**

While we have been developing the optimal ID8 subclone to use for these studies (Aim 1), we have forged ahead with adoptive T cell transfer experiments, similar to what was described in Aim 2 of the original proposal. Our goals are two-fold: (1) to hone our skills at adoptive T cell transfers and flow cytometry, which are technically demanding, and (2) to further refine the experimental questions that will eventually be addressed in the ovarian tumor model. To this end, we have used a well-characterized lymphoma model involving the EL-4 cell line transfected to express the model antigen chicken ovalbumin (OVA). Mice bearing small EL-4/OVA tumors have been infused with CD8+ OT-I TCR transgenic T cells (which recognize OVA, as described in the original proposal). The T cells have been pre-labeled with the vital fluorescent dye CFSE so that they can later be recovered and analyzed by flow cytometry. So far, we have learned that the OT-I T cells undergo a vigorous proliferative response to the EL-4/OVA tumor (Figure 2). Unexpectedly, this occurs without expression of the canonical T cell activation marker CD69, implying that the activation status of these T cells is abnormal (Figure 3). Furthermore, these proliferating T cells fail to express the interleukin-2 (IL-2) receptor alpha chain (CD25), which implies that this is an IL-2-independent proliferative response (Figure 3). By contrast, when non-tumor-bearing mice are vaccinated with OVA protein in complete Freund's adjuvant (which delivers a strong, conventional immune stimulus), the OT-I T cells proliferate equally well but also become positive for CD25 (data not shown). Therefore, we are currently investigating the hypothesis that tumor-derived antigen may trigger an abnormal activation program in T cells that is associated with robust proliferation in the absence of IL-2 signaling, whereas conventional antigen delivery induces proliferation that is associated with IL-2 signaling. If this proves to be the case, we will next evaluate the effect of IL-2 signaling, versus the lack thereof, on the lifespan and functional activity of the CD8+ T cells.

We have also performed adoptive transfer experiments in which OT1 (CD8+) and OT2 (CD4+) T cells were co-administered. In this case, the OT2 T cells appear not to proliferate very well, yet markedly enhance the proliferation of OT1 T cells (not shown). Curiously, the OT1 T cells still remain CD25-negative. Therefore, the 'help' being provided by the CD4+ OT2 T cells appears to be independent of IL-2.

Finally, we have recently initiated experiments to test the effect of the chemotherapeutic drug taxol on the proliferation of OT1 and OT2 T cells. We are interested in learning more about how the chemotherapeutic agents used to treat ovarian cancer affect the T cell response to tumors. The literature is very contradictory on this issue: some studies indicate that chemotherapy enhances anti-tumor immunity, whereas other studies have found impairment of anti-tumor immunity. The OT1/OT2 model we have developed will allow us to rigorously address these issues using defined T cell clones and precise molecular read-outs.

The above studies using the lymphoma model have raised many interesting questions about anti-tumor immunity. Ultimately, we intend to pursue these questions also in the ovarian tumor model, once the optimal ID8 subclone is identified (Aim 1).

**Aim 3: To determine whether tumor-specific CD4+ and CD8+ T cells lacking the *Cbl-b* gene show enhanced functional responses to ovarian tumors.**

Although this aim is not supposed to start until the end of Year 2, we have already received Cbl-b  $-/-$  from Dr. Josef Penninger's lab. Unfortunately, there was a mix-up in their colony and as a result their Cbl-b  $-/-$  mice are no longer on a pure B6 background. Therefore, we are currently backcrossing the mice they sent us onto the B6 background. We are in our third generation, and anticipate needing to do at least three more backcrosses. Therefore, we still expect to be able to complete this aim in the third year.

### **Key Research Accomplishments:**

The following items have been completed or are underway:

**Task 1.** To generate an ovarian tumor cell line that is recognized by antigen-specific CD4+ and CD8+ T cell clones from TCR transgenic mice (Months 1-8). ***\*underway***

- a. Evaluate signaling and transforming properties of epitope-tagged and untagged version of *neu* in cell lines; if problems noted, modify epitopes as needed (Pre-funding period). ***\*completed***
- b. Generate ID8 cell subclones that stably express *neu*<sup>OT1/OT2</sup>; perform in vitro assays to evaluate recognition of OT1 and OT2 epitopes by CD4+ and CD8+ T cells from TCR-transgenic mice (Months 1-4). ***\*completed***
- c. Inject ID8/*neu*<sup>OT1/OT2</sup> cells intraperitoneally into mice to confirm retention of tumorigenic properties and determine optimal level of expression of *neu*<sup>OT1/OT2</sup> (Months 5-8). ***\*underway, modifications were required***

**Task 2.** To define the mechanisms by which ID8 ovarian tumors evade rejection by tumor-specific CD4+ and CD8+ T cells (Months 9-24). ***\*underway***

- a. Generate sufficient numbers of mice bearing tumors expressing *neu*<sup>OT1/OT2</sup> (Months 9-24). ***\*not yet applicable***
- b. Perform immunological studies of adoptively transferred OT1- and OT2-specific T cells and control T cells in mice bearing ovarian tumors expressing *neu*<sup>OT1/OT2</sup>, as per Aim 2 (Months 12-24). ***\*underway using a temporary lymphoma model***

**Task 3.** To determine whether tumor-specific CD4+ and CD8+ T cells lacking the Cbl-b gene show enhanced functional responses to ovarian tumors (Months 21-36). ***\*underway***

- a. Breed OT1, OT2, TEa and P14 TCR transgenes onto the Cbl-b background (Months 21-36). ***\*backcrossing is underway***
- b. Generate sufficient numbers of mice bearing tumors expressing *neu*<sup>OT1/OT2</sup> (Months 21-36). ***\*not yet applicable***
- c. Perform immunological studies of adoptively transferred Cbl-b-deficient OT1- and OT2-specific T cells and control T cells in mice bearing ovarian tumors expressing *neu*<sup>OT1/OT2</sup> (Months 24-36). ***\*not yet applicable***

### **Reportable Outcomes:**

#### **Posters:**

The elements that augment and limit tumor-specific CD8+ lymphocyte responses in vivo. Richard M. Tempero, Marc D. Coltrera, and Brad H. Nelson. Abstract #5497, 93<sup>rd</sup> Annual Meeting of the American Association for Cancer Research, San Francisco CA, April 6-10, 2002.



**Invited presentations:**

Molecular control of T cell proliferation in response to tumors. Brad H. Nelson. Annual Meeting of the British Columbia Cancer Agency, Vancouver, BC, Canada, November 23-24, 2001.

Identifying the signal pathways that drive T-cell proliferation in response to tumors. Brad H. Nelson. 10<sup>th</sup> Annual SPORE Investigators Workshop, Chantilly VA, July 12-16, 2002.

**Conclusions:**

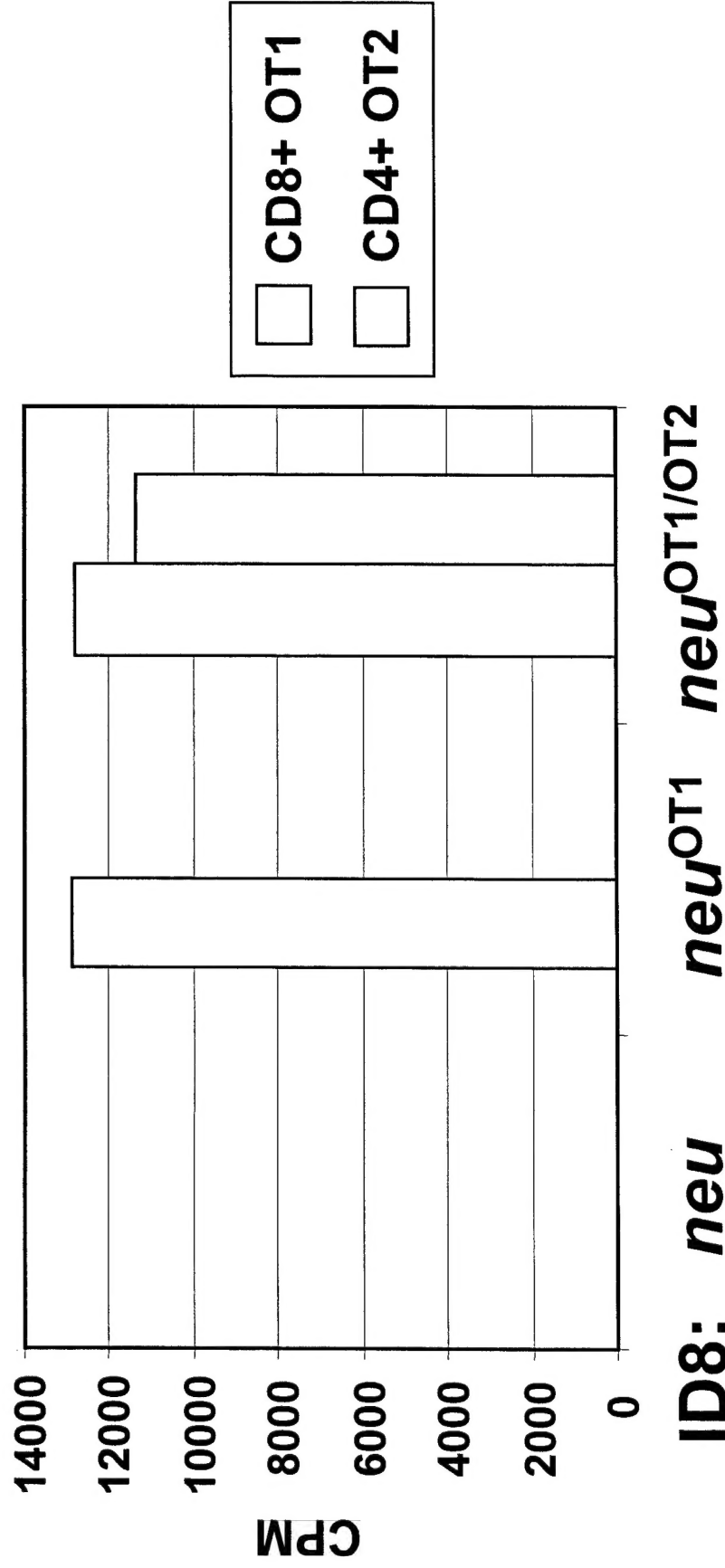
The mouse model we are developing should lead to an improved understanding of the immune response to ovarian cancer and may facilitate the development of novel immune-based therapies or immunopreventive strategies for this disease. Toward this goal, we have now created a dually epitope-tagged version of *neu* that is recognized by the appropriate CD4+ and CD8+ T cells. The generation of ovarian tumor cells expressing *neu*<sup>OT1/OT2</sup> has been somewhat problematic, but we have adopted a sound alternative strategy, and expect to complete Aim 1 in the next year. Adoptive T cell studies have been started using a convenient lymphoma model, and our preliminary results suggest the intriguing possibility that tumors may trigger a different T cell activation program than conventional antigenic stimuli. Finally, we have obtained and are currently backcrossing the Cbl-b <sup>-/-</sup> mice required for Aim 3. No other changes to the research plan are expected.

**References:**

None.

**Appendices:**

See accompanying Figures 1, 2 and 3.



**Figure 1.** Recognition of epitope-tagged *neu* by CD8+ OT1 and CD4+ OT2 TCR transgenic T cells. Splenocytes from OT1 (black bars) or OT2 (gray bars) TCR transgenic mice were incubated with a murine ovarian tumor cell line (ID8) that had been transfected to express either untagged *neu*, *neu* bearing a single OT1 epitope tag (*neu*<sup>OT1</sup>), or dually tagged *neu* (*neu*<sup>OT1/OT2</sup>). Cultures were incubated for 40h, pulsed with tritiated thymidine for 8h, and subjected to liquid scintillation counting.



**Figure 2. OVA-specific CD8<sup>+</sup> T cells proliferate upon recognition of an EL-4 lymphoma expressing OVA antigen. Mice bearing a palpable, subcutaneous OVA-negative (left panels) or OVA-positive (right panels) EL4 lymphoma were infused by tail vein injection with  $5 \times 10^6$  naive OT-I TCR transgenic T cells that were pre-labeled with the fluorescent dye CFSE. Three days later, lymphocytes were isolated from lymph nodes draining the tumor bed, stained with PE-conjugated anti-CD8, and analyzed by flow cytometry. Proliferating T cells can be identified by their diminishing CFSE intensity with each cell division (circled populations of cells). As shown here, OT-I cells proliferate vigorously in response to OVA-positive tumors, but not OVA-negative tumors, demonstrating that this is antigen-induced proliferation.**

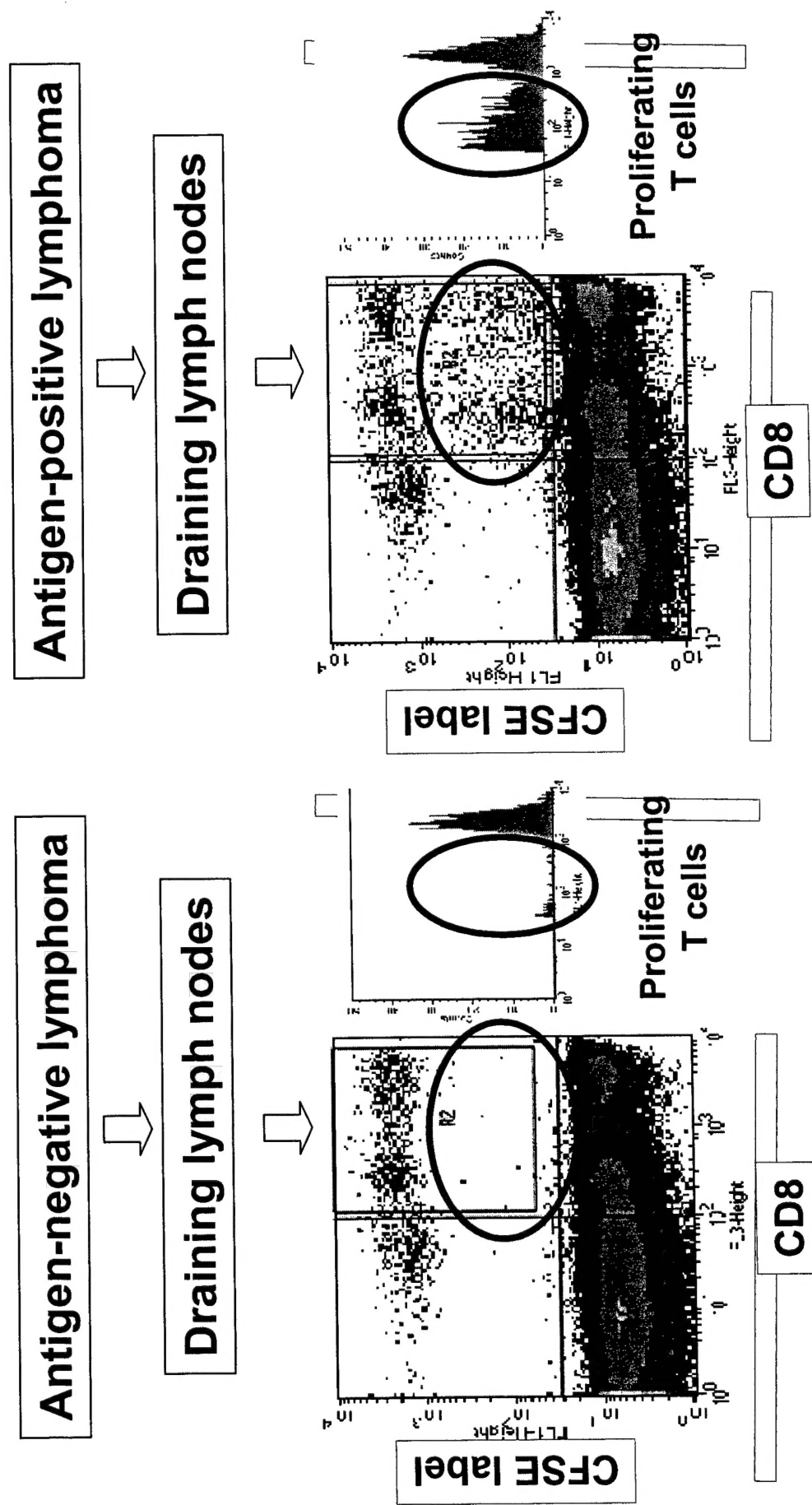


Figure 3. OVA-specific CD8+ T cells show incomplete activation upon tumor recognition. Lymphocytes prepared as in Figure 2, from a mouse bearing an OVA-positive tumor, were further analyzed by flow cytometry for cell size and expression of multiple activation markers. The upper row of panels shows results for the non-proliferating subset, and the lower row refers to the proliferating subset. The results show that proliferating OT-I T cells demonstrate several 'normal' features of activated T cells, including increased cell size, upregulation of CD44, and downregulation of CD62L. However, these cells fail to upregulate IL-2Ralpha or CD69 expression, leading to the conclusion that they are abnormally or incompletely activated.

